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(57) Abstract

Novel protein tyrosine phosphatases in which the invariant aspartate residue is replaced with an alanine residue and which bind to a tyrosine phosphorylated substrate and are catalytically attenuated are described. Also described are methods of identifying tyrosine phosphorylated proteins which complex with the described protein tyrosine phosphatases.

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SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASES

Background of the Invention

The protein tyrosine phosphatase (PTP) family of enzymes consists of more than 500 structurally diverse proteins which have in common the highly conserved 250 amino acid PTP catalytic domain, but which display considerable variation in their non-catalytic segments (Charbonneau and Tonks, Annu. Rev. Cell Biol. 8:463-493 (1992); Tonks, Semin. Cell Biol. 4:373-453 (1993)). This structural diversity presumably reflects the diversity of physiological roles of individual PTP family members, which in certain cases have been demonstrated to have specific functions in growth, development and differentiation (Desai et al., Cell 84:599-609 (1996); Kishihara et al., Cell 74:143-156 (1993); Perkins et al., Cell 70:225-236 (1992); Pingel and Thomas, Cell 58:1055-1065 (1989); Schultz et al., Cell 73:1445-1454 (1993)). Although recent studies have also generated considerable information regarding the structure, expression and regulation of PTPs, the nature of the tyrosine phosphorylated substrates through which the PTPs exert their effects remains to be determined. Studies with a limited number of synthetic phosphopeptide substrates have demonstrated some differences in substrate selectivity of different PTPs (Cho et al., Protein Sci. 2: 977-984 (1993); Dechert et al., Eur. J. Biochem. 231:673-681 (1995)), and have indicated preferences for certain amino acid residues at particular positions around the phosphorylated tyrosine residue (Ruzzene et al., Eur. J. Biochem. 211:289-295 (1993); Zhang et al., Biochemistry 33:2285-2290 (1994)). This indicates that PTPs display a certain level of substrate selectivity in vitro, although

the physiological relevance of the substrates used in these studies is unclear.

Summary of the Invention

As described herein, the substrate specificity of mammalian protein tyrosine phosphatases (PTPs) has been investigated using a novel substrate trapping approach in which mutant or altered forms of the mammalian PTP, also referred to as substrate trapping PTPs, are used to bind (trap) one or more substrates of the PTP. Binding of the substrate trapping PTP with a substrate of the PTP results in the formation of a complex which can be readily observed, and, if desired, isolated, and characterized. The mutant forms of the PTPs have attenuated catalytic activity (lack catalytic activity or have reduced catalytic activity) relative to the wild type PTP but retain the ability to bind tyrosine phosphorylated substrate(s) of the wild type PTP.

The methods of the present invention are specifically exempliefied herein with respect to the phosphatases PTP1B and PTP-PEST; however, it is understood that the invention is not limited to these specific PTPs but is applicable to all members of the PTP family. In order to identify potential substrates of PTP1B and PTP-PEST, mutant (i.e., altered or substrate trapping) forms of PTP1B and PTP-PEST were generated which were catalytically attenuated but retained the ability to bind substrates. These mutant PTPs associated in stable complexes with proteins which were identified by immunoblotting as p210 bcr:abl and $p130^{cas}$, respectively. These associations were observed in lysates from several cell lines and in transfected COS cells, indicating that p210 bcr:abl and $p130^{cas}$ represent major physiologically relevant substrates for PTP1B and PTP-PEST.

These results provide the first demonstration of PTPs having inherently restricted substrate specificity in vivo.

The methods used to identify p210 bcr:abl and $p130^{cas}$ as specific substrates for PTP1B and PTP-PEST, respectively, are generally applicable to any member of the PTP family, of which approximately 500 members have currently been reported, and can be used to determine the physiological substrates of other members of the PTP family.

One embodiment of the invention relates to novel mutant PTPs in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}). These PTPs retain the ability to form a complex with, or bind, their tyrosine phosphorylated substrates, but are catalytically attenuated. In one embodiment, the invention relates to the phosphatase PTP1B in which the invariant aspartate residue at position 181 is replaced with alanine (D181A). In another embodiment the invention relates to the phosphatase PTP-PEST in which the invariant aspartate residue at position 199 is replaced with an alanine (D199A). Another embodiment of the invention relates to a PTP-PEST phosphatase in which the cysteine residue at position 231 is replaced with a serine (C231S). The invention also relates to other mutant or substrate trapping PTPs in which the invariant aspartate residue is replaced with or changed to another amino acid residue, such as alanine. The invariant aspartate residue can be identified in other PTPs by aligning the PTP nucleotide sequence with the nucleotide sequence of a PTP for which the location of the invariant aspartate residue is known.

The invention also relates to a method of identifying a tyrosine phosphorylated substrate of a protein tyrosine phosphatase. According to one embodiment of the present invention, a tyrosine phosphorylated protein of interest is combined with one or more PTP(s) in which the invariant aspartate residue is replaced with an amino acid which does

not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}), and the presence or absence of a complex between the protein and the PTP(s) is determined. Presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the PTP. The PTP DA mutant binds to or complexes with its substrate but does not dephosphorylate it (or does so very slowly), thereby allowing the complex to be observed and, optionally, isolated and identified. In a particular embodiment of the invention, the invariant aspartate is replaced with an alanine residue (a PTP DA mutation or alteration)

In an alternative embodiment of the present invention, a PTP of interest in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}), is combined with one or more tyrosine phosphorylated proteins, and the presence or absence of a complex between the protein(s) and the PTP is determined. Presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the PTP. The PTP DA mutant binds to or complexes with its substrate but does not dephosphorylate it (or does so very slowly), thereby allowing the complex to be observed, and, optionally, isolated and identified. In one embodiment of the invention, the invariant aspartate residue is replaced with an alanine residue (a PTP DA mutation or alteration)

The present invention also relates to a method of identifying a tyrosine phosphorylated substrate of a protein tyrosine phosphatase wherein more than one tyrosine phosphorylated protein of interest is combined with more than one PTP of interest in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which

results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., the invariant aspartate is replaced with an alanine residue). Complexes formed in the combination can be isolated and the component PTP and substrate can be identified.

The invention also pertains to a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal a PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., the invariant aspartate is replaced with an alanine residue) and which forms a complex with the tyrosine phosphorylated protein. The PTP mutant binds to the phosphorylated protein without dephosphorylating it, thereby inhibiting the activity of the protein and reducing its downstream effects.

For example, the invention relates to a method of reducing the transforming effects of oncogenes associated with $p130^{cas}$, a substrate of PTP-PEST, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. Wild type PTP-PEST binds and dephosphorylates $p130^{cas}$, thereby negatively regulating its downstream effects. DA mutants of PTP-PEST bind but do not dephosphorylate $p130^{cas}$ (or dephosphorylate it at a reduced rate); the substrate is thus tied up in the complex with the substrate trapping form of PTP-PEST and cannot exert its downstream effects. Similarly, the invention relates to a method of reducing the formation of signalling complexes associated with $p130^{cas}$, particularly those signalling complexes which induce mitogenic pathways, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue.

The present invention also relates to assays for identifying agents which alter, e.g., enhance or inhibit, the interaction between a PTP and its phosphorylated substrate. Agents identified by these assays can be agonists (e.g., agents which enhance or increase the activity of the PTP) or antagonists (e.g., agents which inhibit or decrease the activity of the PTP) of PTP activity. The agent may be an endogenous physiological substance or may be a natural or synthetic drug, including small organic molecules.

For example, the tyrosine phosphorylated substrate of a PTP can be identified by the methods described herein. An enzymatic activity assay utilizing the wild type PTP can be carried out in the presence of an agent to be tested, and the resulting amount of enzyme activity can be compared with the amount of enzyme activity in the absence of the agent to be tested. A decrease in the enzymatic activity in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in the enzymatic activity in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Alternatively, a competitive binding assay can be carried out utilizing the mutant PTP in the presence of an agent to be tested, and the resulting extent of binding of the mutant PTP to its substrate can be compared with the extent of binding in the absence of the agent to be tested. A decrease in the extent of binding in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Thus, the compositions and methods described herein are useful in identifying the tyrosine phosphorylated

substrates of members of the PTP family of phosphatases, as well as in regulating the activity of identified substrates. The compositions and methods described herein are also useful for identifying tyrosine phosphorylated proteins which are related to a particular disease or disorder, and to methods of screening for modulators which enhance or inhibit the PTP/substrate interaction for use in therapeutic applications.

Brief Description of the Drawings

Figures 1A and 1B show a multiple sequence alignment of the catalytic domains of PTPs. In Figure 1A, cytosolic eukaryotic PTPs and domain 1 of RPTPs are combined into one group, domains 2 of RPTPs are in a second group, and the *Yersinia* PTP is in a third. Invariant residues shared among all three groups are shown in red. Invariant and highly conserved residues within a group are shown in blue and green, respectively. Within the Yersinia PTP sequence, residue that are either invariant or highly conserved between the cytosolic and RPTP domain sequences are colored blue and green, respectively. The position of residues of PTP1B that interact with the peptide are indicated with a red arrow, and the residue numbering at the bottom of the alignment corresponds to that for PTP1B. Figure 1B is a black and white photocopy of Figure 1A in which the colored areas are indicated with labeled arrows.

Figure 2 shows the Vmax and Km of various PTP1B mutants toward RCML.

Detailed Description of the Invention

The PTP family of enzymes contains a common evolutionarily conserved segment of approximately 250 amino acids known as the PTP catalytic domain. Within this

conserved domain is a unique signature sequence motif, [I/V]HCXAGXXR[S/T]G, that is invariant among all PTPs. The cysteine residue in this motif is invariant in members of the family and is known to be essential for catalysis. It functions as a nucleophile to attack the phosphate moiety of the incoming substrate. If the cysteine residue is altered by site-directed mutagenesis to serine (CS mutants) or alanine (CA mutants), the resulting PTP is catalytically attenuated but retains the ability to complex with, or bind, its substrate, at least in vitro. These results have been confirmed relative to MKP-1, a member of the PTP family (Sun et al., Cell 75:487-493 (1993)), as well as other PTPs. However, although these CS mutants can in general bind effectively to phosphotyrosyl substrates in vitro, in many cases such complexes cannot be isolated in vivo. Thus, the CS mutants are limited in their applicability and cannot be used to isolate all combinations of PTPs and substrates.

The crystal structures of PTP1B alone (Barford, et al., Science 263:1397-1404 (1994)) and in a complex with a phosphotyrosine-containing peptide (Jia et al., Science 268:1754-1758 (1995)) were recently determined. These structures indicated twenty seven invariant residues (Barford et al., 1994), one of which is an aspartate residue. This aspartate residue is invariant across the catalytic domains of PTP family members. That is, if the amino acid sequences of the PTP family members are aligned, the aspartate residue is present in each PTP at a corresponding location, although the position numbers may be different due to the shifts required to maximize alignment (see the Figure (from Barford et al., Nature Struc. Biol. 2:1043-1053 (1995)) for an alignment of various PTP sequences). Sequences for which the alignment has not yet been published can readily be aligned with other known PTP sequences, e.g., utilizing available computer software such as GENEWORKS.

Thus, mutant PTPs other than those specifically described herein can readily be made by aligning the amino acid sequence of the PTP catalytic domain with those described herein, identifying the invariant aspartate residue, and changing the residue by site-directed mutagenesis. Although the specific examples of PTP mutants described herein are aspartate to alanine mutants (DA mutants), it is understood that the invention is not limited to changes of aspartate to alanine. The invariant aspartate residue can be changed, e.g., by site-directed mutagenesis, to any amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}). For example, the invariant aspartate residue can be changed or mutated to an alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine or histidine.

As described herein, pervanadate-treated cells were used as an abundant source of tyrosine phosphorylated proteins to investigate the substrate specificity of PTP-PEST. PTP-PEST is an 88 kDa cytosolic PTP (Charest et al., Biochem. J. 308:425-432 (1995); den Hertog et al., Biochem. Biophys. Res. Commun. 184:1241-1249 (1992); Takekawa et al., Biochem. Biophys. Res. Commun. 189:1223-1230 (1992); Yang et al., J. Biol. Chem. 268:6622-6628 (1993); Yang et al., J. Biol. Chem. 268:17650 (1993)) which is expressed ubiquitously in mammalian tissues (Yi et al., Blood 78: 2222-2228 (1991)), and which exhibits high specific activity when assayed in vitro using artificial tyrosine phosphorylated substrates (Garton and Tonks, EMBO J. 13:3763-3771 (1994)). It has previously been demonstrated that PTP-PEST is subject to regulation via phosphorylation of Ser39 in vitro and in vivo. This modification is catalyzed by both protein kinase C (PKC) and protein kinase

A (PKA), and results in reduced enzyme activity as a consequence of an increase in the Km of the dephosphorylation reaction (Garton and Tonks, EMBO J. 13:3763-3771 (1994)). It appears likely that further regulatory mechanisms exist for PTP-PEST, since this enzyme would be expected to exert a considerable negative influence on the tyrosine phosphorylation state of cytosolic substrates of tyrosine kinases. One possibility is that this influence could be limited by the substrate specificity of PTP-PEST.

The substrate specificity of PTP1B was investigated utilizing the same methods outlined for PTP-PEST, with the exception that the cells were not treated with pervanadate. A combination of in vitro dephosphorylation and substrate trapping experiments were used to study the substrate interactions of PTP1B and PTP-PEST. The substrate trapping methods outlined herein are generally applicable to any PTP by virtue of the shared invariant aspartate residue, and should therefore prove useful in delineating the substrate preference of other PTP family members. In particular, the use of mutant, catalytically impaired PTPs to trap, and thereby isolate, potential substrates will greatly facilitate the identification of physiologically important substrates for individual PTPs, leading to improved understanding of the roles of these enzymes in regulation of cellular processes.

One embodiment of the invention relates to novel PTPs in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}). These PTPs retain the ability to form a complex with, or bind, their tyrosine phosphorylated substrates but are catalytically attenuated. As defined herein, "attenuated" activity is intended to mean that the phosphatase retains a

similar Km to that of the wild type phosphatase but has a Vmax which is reduced by a factor of at least 10^4 relative to the wild type enzyme. This includes catalytic activity which is either reduced or abolished relative to the wild type PTP. For example, the invariant aspartate residue can be changed or mutated to an alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine or histidine.

The novel PTPs described herein, in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}), can also comprise other mutations, particularly those which assist in stabilizing the PTP/substrate complex. For example, a mutation of the [serine/threonine] residue in the signature motif to an alanine residue changes the rate-determining step of the dephosphorylation reaction from the formation of the transition state to the break down of the transition state, thereby stabilizing the PTP/substrate complex. Such mutations may be valuably combined with the replacement of the invariant aspartate residue, particularly assisting in stabilizing the complex and facilitating the observation and isolation of the complex.

PTPs suitable for use in the invention include any PTP which has an invariant aspartate residue in a corresponding position. As defined herein, a phosphatase is a member of the PTP family if it contains the signature motif [I/V]HCXAGXXR[S/T]G. Dual specificity PTPs, i.e., PTPs which dephosphorylate both phosphorylated tyrosine and phosphorylated serine or threonine, are also suitable for use in the invention. Appropriate PTPs include, but are not limited to, PTP1B, PTP-PEST, PTP_γ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10 and PTPH1.

In one embodiment, the invention relates to the phosphatase PTP1B in which the aspartate residue at position 181 is replaced with alanine (D181A). In another embodiment the invention relates to the phosphatase PTP-PEST in which the invariant aspartate residue at position 199 is replaced with an alanine (D199A). Another embodiment of the invention relates to a PTP-PEST phosphatase in which the cysteine residue at position 231 is replaced with a serine (C231S).

The invention also relates to a method of identifying a tyrosine phosphorylated protein which is a substrate of a particular protein tyrosine phosphatase. According to one embodiment of the present invention, a tyrosine phosphorylated protein of interest is combined with at least one PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an alanine residue), and the presence or absence of a complex between the protein and the PTP is determined. Presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the PTP. The PTP DA mutant (substrate trapping mutant) binds to or complexes with its substrate but does not dephosphorylate it (or does so very slowly), thereby allowing the complex to be isolated and identified.

The phosphorylated protein/PTP complex may be isolated by conventional isolation techniques as described in U.S. Patent No. 5,352,660 to Pawson, including salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. Furthermore, to facilitate the determination of the presence of the protein/PTP complex, antibodies against the PTP or the phosphorylated protein can be used, as well as labelled

PTPs and/or labelled phosphorylated substrates. The PTP or phosphorylated protein can be labelled with various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include, but are not limited to, horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase and acetylcholinesterase. Examples of suitable fluorescent materials include, but are not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin. Appropriate luminescent materials include luminol, and suitable radioactive material include radioactive phosphorous ^{32}P, iodine I^{125}, I^{131} or tritium.

Alternatively, the invention pertains to a method of identifying a tyrosine phosphorylated protein which is a substrate of a PTP, comprising combining a PTP of interest in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an alanine residue), with at least one tyrosine phosphorylated protein, thereby producing a combination; and determining the presence or absence of a complex in the combination, wherein presence of a complex in the combination between a tyrosine phosphorylated protein and the PTP indicates that the tyrosine phosphorylated protein is a substrate of the PTP.

The substrate trapping PTPs of the present invention can also be used in place of wild type PTPs to screen phosphotyrosyl peptide libraries for peptides which bind to the PTP as described in Songyang et al. (Nature 373:536-539 (1995); Cell 72:767-778 (1993)). Peptides identified from such peptide libraries can then be assessed to determine whether tyrosine phosphorylated proteins containing these peptides exist in nature.

Any tyrosine phosphorylated protein is suitable as a potential substrate in the present invention. Tyrosine phosphorylated proteins are well known in the art. Specific examples of appropriate substrates include, without limitation, $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor. Of particular interest are tyrosine phosphorylated proteins which have been implicated in a mammalian disease or disorder.

The invention also pertains to a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal a PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an alanine residue) and which forms a complex with the tyrosine phosphorylated protein. The PTP DA mutant binds to the phosphorylated protein without dephosphorylating it (or causing dephsophorylation at a greatly reduced rate), thereby inhibiting the activity of the protein and reducing its downstream effects. As used herein, "reducing" includes both reduction and complete abolishment, e.g., of one or more activities or functions of the phosphorylated protein.

For example, the invention relates to a method of reducing the transforming effects of oncogenes associated with $p130^{cas}$, a substrate of PTP-PEST, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. Wild type PTP-PEST binds and dephosphorylates $p130^{cas}$, thereby negatively regulating its downstream effects. DA mutants of PTP-PEST bind but do not dephosphorylate $p130^{cas}$ (or do so at a greatly reduced rate); the substrate is thus tied up in the complex with the substrate trapping form of PTP-PEST and cannot exert its downstream effects. Similarly, the invention relates to a

method of reducing the formation of signalling complexes associated with $p130^{cas}$, particularly those signalling complexes which induce mitogenic pathways, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. The PTP binds to and/or dephosphorylates $p130^{cas}$, thereby negatively regulating the downstream effects of $p130^{cas}$ and reducing the formation of signalling complexes associated with $p130^{cas}$.

The substrate trapping mutant PTPs of the present invention can be used in virtually any application in place of, or in addition to, a corresponding wild type PTP. The advantages of such a utility lie in the ability of the mutant PTP to mimic the function of the wild type enzyme, e.g., to decrease the activity of its tyrosine phosphorylated substrate, without inducing the harmful cytotoxic effects commonly observed with administration or overexpression of the wild type PTP. Thus, the invention also pertains to a method of reducing the cytotoxic effects associated with administration or overexpression of wild type PTPs. For example, CS mutants of MKP-1 have been shown to have the same functional effect as wild type MKP-1 without induction of potentially harmful side effects. Thus, PTPs described herein, in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an alanine residue), can be used in many applications in place of the corresponding wild type enzyme. As used herein, a "corresponding" enzyme is one which is the same as the mutant PTP (e.g., PTP-PEST and PTP-PEST D199A) or one which is different from the mutant PTP but recognizes the same substrate as the mutant PTP.

The mutant PTPs described herein can also be used therapeutically to reduce the activity of a tyrosine phosphorylated protein, such as by a gene therapy method in which the mutant PTP described herein, or a functional portion thereof which retains the ability to bind to its tyrosine phosphorylated substrate, is introduced into a subject and in whom the mutant PTP is expressed. The mutant PTP replaces, either partially or totally, the wild type enzyme which is normally produced or competes with the wild type PTP for binding to the substrate. For example, a specific tyrosine phosphorylated protein can be identified which is implicated in a particular disease or disorder (such as a protein tyrosine kinase). At least one PTP which acts to dephosphorylate the selected tyrosine phosphorylated protein of the present invention can be identified by the methods described herein. The wild type or mutant form of the PTP can be administered to a subject in need of treatment in order to tie up or bind the tyrosine phosphorylated substrate, thereby inhibiting or reducing the function of the phosphorylated protein. In a preferred embodiment, the mutant PTP is administered in place of the wild type enzyme in order to reduce the cytotoxic effects associated with overexpression of the wild type enzyme. Procedures for gene therapy are known in the art (see U.S. Patent No. 5,399,346 to Anderson et al.) and can be modified by methods known in the art to appropriately express the specific mutant and wild type PTPs of the present invention.

The present invention also relates to assays for identifying agents which alter, e.g., enhance or inhibit, the interaction between a PTP and its phosphorylated substrate. Agents identified by these assays can be agonists (e.g., agents which enhance or increase the activity of the PTP) or antagonists (e.g., agents which inhibit or decrease the activity of the PTP) of PTP activity. The agent may be an endogenous physiological

substance or may be a natural or synthetic drug, including small organic molecules.

For example, the tyrosine phosphorylated substrate of a PTP can be identified by the methods described herein. An enzymatic activity assay utilizing the wild type PTP can be carried out in the presence of an agent to be tested, and the resulting amount of enzyme activity can be compared with the amount of enzyme activity in the absence of the agent to be tested. Enzymatic activity assays are known in the art; for example, assays of PTP activity using a tyrosine phosphorylated ^{32}P-labelled substrate are described in Flint et al. (EMBO J. 12:1937-1946 (1993)). A decrease in the enzymatic activity in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in the enzymatic activity in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Alternatively, a competitive binding assay can be carried out utilizing the mutant PTP in the presence of an agent to be tested, and the resulting extent of binding of the mutant PTP to its substrate can be compared with the extent of binding in the absence of the agent to be tested. Competitive binding assays are known in the art; for example, U.S. Patent No. 5,352,660 to Pawson describes methods suitable for use in this invention. A decrease in the extent of binding in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

According to the present invention, tyrosine phosphorylated peptides identified with mutant PTPs from peptide libraries by the methods of Songyang et al. (Nature

373:536-539 (1995); Cell 72:767-778 (1993)) can be used herein in place of the complete tyrosine phosphorylated protein in competitive binding assays.

The present invention also pertains to pharmaceutical compositions comprising a PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an alanine residue). For instance, the PTP of the present invention can be formulated with a physiologically acceptable medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists, and will depend on the ultimate pharmaceutical formulation desired. Methods of introduction of exogenous PTPs at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal. Other suitable methods of introduction can also include rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated by reference in their entirety.

EXAMPLES

Materials and Methods

The following is a description of the materials and methods used in the work described herein.

Generation, Expression and Purification of Mutant PTP Proteins

Point mutations within the catalytic domains of PTP-PEST (D199A, C231S) and PTP1B (D181A, C215S) were introduced by site-directed mutagenesis using the Muta-Gene™ in vitro mutagenesis kit (Bio-Rad, Richmond, CA). Regions containing the required point mutation were then exchanged with the wild type sequences within appropriate expression vectors, and the replaced mutant regions were sequenced in their entirety to verify the absence of additional mutations.

Full length PTP-PEST proteins (wild type and mutant proteins, containing either Asp199 to Ala or Cys231 to Ser mutations) and the wild type PTP-PEST catalytic domain (amino acids 1-305) were expressed in Sf9 cells using recombinant baculovirus (BaculoGold™, Pharmingen, San Diego, CA), and purified as described in Garton and Tonks (EMBO J. 13:3763-3771 (1994)). Truncated forms of wild type and mutant PTP-PEST proteins, comprising amino acid residues 1-305 of PTP-PEST were also expressed in E. coli as GST fusion proteins following subcloning of PTP-PEST DNA in-frame downstream of GST in pGEX vectors (Pharmacia Biotech Inc., Uppsala, Sweden). Twenty-five ml of E. coli transformed with the appropriate vector were grown to log phase (OD_{600} approximately 0.5). Fusion protein expression was then induced by addition of 0.2 mM isopropyl-1-thio-b-D-galactopyranoside, and the cells were grown for 2-4 hours at 30°C. Cells were harvested by centrifugation, incubated with 50 mg/ml lysozyme in 3 ml buffer containing 50 mM Tris-HCl, pH 7.4, 5mM EDTA, 1 mM PMSF, 1 mM benzamidine, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 0.1 % Triton X-100 and 150 mM

NaCl, then lysed by sonication (3 x 10s). Following removal of insoluble material by centrifugation (20 minutes at 300,000 x *g*), fusion proteins were isolated by incubation for 30 min at 4°C with 100 ml glutathione-Sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden), and the beads were then collected by centrifugation and washed three times with buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 % glycerol, 1 % Triton X-100 and 100 mM NaCl). This procedure yielded essentially homogeneous fusion protein at a concentration of 1 mg protein/ml glutathione-Sepharose beads. PTP1B proteins (wild type and mutant forms) comprising amino acids 1-321 were expressed in E.coli and purified to homogeneity as described in Barford et al. (J. Mol. Biol. 239:726-730 (1994)).

Cell Culture, Transfection, Preparation of Lysates and Fractionation

HeLa and COS cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% fetal bovine serum (FBS); Wi38, C2C12 and MvLu cells were grown in DMEM containing 10% FBS; 293 cells were grown in DMEM containing 10% calf serum; MCF10A cells were grown in 50% DMEM, 50% Ham's F-12 containing 5% horse serum, 20 ng/ml epidermal growth factor, 10 mg/ml insulin, 0.5 mg/ml hydrocortisone and 0.25 mg/ml fungizone. All media also contained penicillin and streptomycin at 100 U/ml and 100 mg/ml, respectively, and all cells were grown at 37°C. Calcium phosphate-mediated transfection was used to introduce cDNA encoding wild type and mutant PTP-PEST proteins into COS cells. These were encoded by PTP-PEST cDNA subcloned into the plasmid PMT2, from which expression was driven by an adenovirus major late promoter; 20 mg DNA was used for transfection of each 10 cm plate of cells. The level of expression of PTP-PEST constructs was similar in all cases.

Prior to cell lysis, 70-90% confluent cultures of cells were treated for 30 minutes with 0.1 mM pervanadate (20 ml of a fresh solution containing 50 mM sodium metavanadate ($NaVO_3$) and 50 mM H_2O_2 were added to 10 ml medium). Treatment of cells with H_2O_2 and vanadate leads to a synergistic increase in phosphotyrosine levels, presumably due to inhibition of intracellular PTPs by vanadate. The synergism between H_2O_2 and vanadate has previously been suggested to result from improved accumulation of the resultant oxidized vanadate (pervanadate) within the cells when compared to vanadate itself (Heffetz et al., J. Biol. Chem. 265:2896-2902 (1990)). It is important to note that during the preparation of cell lysates, dilution occurs such that the inhibitory effect of vanadate on PTP action is lost. Pervanadate treatment resulted in the appearance of at least 50 prominent phosphotyrosine protein bands in all cell types, whereas untreated cells contained virtually undetectable levels of phosphotyrosine (data not shown).

Cells were lysed in Buffer A containing 5 mM iodoacetic acid, which was included in order to inhibit irreversibly cellular PTPs. Following incubation at 4°C for 30 minutes, 10 mM DTT was added to inactivate any unreacted iodoacetic acid. Insoluble material was then removed by centrifugation for 20 minutes at 300,000 x *g*. The resultant lysates were stable with regard to their phosphotyrosine content during long term (several months) storage at -70°C and during prolonged (at least 20 hours) incubation at 4°C, in the absence of exogenous added PTPs.

Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography using a Mono Q FPLC column (Pharmacia). The sample (50 mg total protein at 3 mg/ml in buffer A) was diluted in three volumes of buffer B (20 mM tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 0.1% Triton X-100) prior to

loading. Proteins were eluted at a flow rate of 1 ml/min with a linear gradient of 0-0.5 M NaCl in buffer B over 20 fractions (1 ml fraction volume), followed by a second gradient of 0.5-1.0 M NaCl in buffer B over 5 fractions. Phosphotyrosine-containing proteins were detected within fractions 7-21 according to anti-phosphotyrosine immunoblotting. The same procedures were followed for PTP1B, with the exception that the cells were not treated with pervanadate.

Dephosphorylation Reactions

Lysates of pervanadate-treated HeLa cells (1-2 mg protein/ml) containing tyrosine phosphorylated proteins were incubated on ice in the absence or presence of purified active PTPs at a concentration of 2 nM. Dephosphorylation was terminated by the removal of aliquots (30 mg protein) into SDS-PAGE sample buffer, and the extent of dephosphorylation was determined by immunoblotting using the monoclonal antibody G104. Assays of PTP activity using tyrosine phosphorylated ^{32}P-labelled RCM-lysozyme as substrate were performed as described in Flint et al. (EMBO J. 12:1937-1946 (1993)).

Antibodies and Immunoblotting

The PTP-PEST monoclonal antibody AG25 was raised against baculovirus-expressed purified full-length PTP-PEST. The anti-phosphotyrosine monoclonal antibody G104 was generated using as antigen phosphotyrosine, alanine and glycine, in a 1:1:1 ratio, polymerized in the presence of keyhole limpet hemocyanin with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide, a method originally described in Kamps and Sefton (Oncogene 2:305-315 (1988)). p130^{cas} monoclonal antibody was from Transduction Laboratories (Lexington, Ky). Monoclonal antibody FG6 against PTP1B was provided by Dr David Hill (Calbiochem

Oncogene Research Products, Cambridge, MA). Visualization of proteins by immunoblotting was achieved by enhanced chemiluminescence (ECL) using HRP-conjugated secondary antibodies (Amersham Life Science Inc., Arlington Heights, Il) and the SuperSignal™ CL-HRP substrate system (Pierce, Rockford, Il).

Immunoprecipitation and Substrate Trapping

Immunoprecipitation of PTP-PEST from transfected COS cells was performed following covalent coupling of monoclonal antibody AG25 to protein A-Sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden) using the chemical cross-linking agent dimethyl pimelimidate (Schneider et al., J. Biol. Chem. 257:10766-10769 (1982)). Antibody was first bound to protein A-Sepharose at a concentration of 1 mg/ml bead volume, and unbound material was then removed by three washes with 0.2 M sodium borate, pH 9. Covalent coupling was achieved by incubation at room temperature for 30 minutes in the presence of 20 mM dimethyl pimelimidate in 0.2 M sodium borate, pH 9. The beads were then incubated for 1 hour with an excess of 0.2 M ethanolamine, pH 8, to block any unreacted cross-linker, and washed three times with PBS prior to storage at 4°C. Ten ml of AG25 beads were used to precipitate transfected PTP-PEST from lysates containing approximately 0.375 mg protein.

Substrate trapping was performed using various PTP affinity matrices. The full-length PTP-PEST matrix utilized covalent coupled AG25-protein A-Sepharose beads to which purified baculovirus-expressed PTP-PEST protein was bound. Aliquots (10ml) of AG25 beads were incubated for 2 hours at 4°C in 100 ml buffer A in the presence of 5 mg of purified PTP-PEST (wild type or mutant forms); unbound PTP-PEST was then removed by washing three times with 1 ml buffer A. The resultant PTP-PEST-AG25-protein A-Sepharose beads contained approximately 2 mg of PTP-PEST per 10 ml aliquot. Substrate

trapping was also carried out with glutathione-Sepharose beads bound to bacterially-expressed GST fusion proteins containing the catalytic domain of PTP-PEST.

PTP1B was also used in substrate trapping experiments. In this case, the monoclonal antibody FG6 was precoupled to protein A-Sepharose in the absence of cross-linker (2 mg antibody/10 ml beads), then purified PTP1B proteins were added in excess and incubated at 4°C for 2 hours. Following removal of unbound PTP1B, 10 ml beads contained approximately 2 mg PTP1B.

Pervanadate-treated cell lysates, or column fractions, were used as a source of phosphotyrosine-containing proteins for substrate trapping experiments. In general, lysates containing 0.25-0.5 mg protein in 0.5 ml buffer A (including 5 mM iodoacetic acid, 10 mM DTT) were incubated at 4°C for 2 hours in the presence of 10 ml of affinity matrix containing approximately 2 mg of the appropriate PTP protein. Unbound proteins were then removed from the samples by washing three times with 1 ml buffer A, and bound material was collected by addition of 50 ml SDS-PAGE sample buffer followed by heating at 95°C for 5 minutes; proteins bound to the beads were then analyzed by SDS-PAGE followed by immunoblotting.

Results

The following details the results of the work described herein carried out as described above.

PTP1B and p210 bcr:abl

Chronic myelogenous leukemia (CML) is a clonal disorder of the haematopoietic stem cell that is characterized by the Philadelphia chromosome, in which the c-Abl proto-oncogene on chromosome 9, encoding a PTK, becomes linked to the bcr gene on chromosome 22. This results in the generation of a bcr:able fusion protein, p210 bcr:abl, in which the PTK activity is enhanced relative to

that of c-Abl. Current data indicates that this cytogenetic abnormality is the primary and sole causative event in CML. Expression of p210 bcr:abl produces abnormal patterns of tyrosine phosphorylation that result in the aberrant maturation of the haematopoietic stem cell that is characteristic of CML.

Expression of PTP1B mRNA and protein is enhanced as a consequence of p210 bcr:abl expression in Rat1, Mo7 and BaF3 cells. Changes in PTP1B activity, which were commensurate with the change in enzyme protein, were also observed. These changes are specific for PTP1B and are not seen in closely related homologue (65% identity) TC-PTP or in other tested PTPs, including SHP-1, SHP-2 and PTP-PEST. The increase in expression of PTP1B was also observed in Ph+ B-lymphoid cells derived from a CML patient relative to Ph- cells from the same patient.

The changes in PTP1B levels were induced specifically by p210 bcr:abl and were not seen in cells expressing other PTKs including v-abl, v-src or other oncoproteins such as myc. The PTK activity of p210 bcr:abl was essential for the increase in expression of PTP1B, since expression of an inactive lysine to arginine mutant form of p210 bcr:abl in Rat1 cells did not alter PTP1B levels. The increase in PTP1B levels is a rapid response to induction of p210 bcr:abl. When BaF3 cells expressing a temperature-sensitive mutant form of p210 bcr:abl were shifted to the permissive temperature for the PTK, PTP1B levels were observed to increase within 12-24 hours coincident with the appearance of the active form of the PTK. THese data indicate that the alteration PTP1B levels is a relatively rapid response to the appearance of p210 bcr:abl, rather than a long-term adaptive response of the cells.

In transient cotransfection experiments in COS cells, PTP1B dephosphorylates p210 bcr:abl but not v-abl. When the PTP1B D181A mutant was expressed as a GST fusion protein,

purified and incubated with lysates of Mo7-p210 cells (which overexpress p210 bcr:abl), a complex of the mutant PTP and p210 bcr:abl was isolated. In contrast, tyrosine phosphorylated c-abl, which was also present in the lysates, did not bind to the mutant PTP. The interaction between PTP1B D181A and p210 bcr:abl was blocked by vanadate, suggesting that the interaction involved the active site of the PTP.

Following transient coexpression in COS cells, PTP1B D181A formed a complex with p210 bcr:abl. Preliminary data indicate that the Y177F mutant form of p210 bcr:abl did not interact with PTP1b D181A, suggesting that this tyrosine residue is a component of the binding site in the PTK. This tyrosine residue in p210 bcr:abl is phosphorylated in vivo and has been demonstrated to serve as a docking site for GRB2. Direct interaction of the pTyr in p210 bcr:abl and the SH2 domain of GRB2 is essential for the transforming activity of the PTK. Interaction of PTP1B D181A with p210 bcr:abl interferes with the association of the PTK with GRB2. Taken together, these data suggest that p210 bcr:abl is a physiological substrate of PTP1B and that PTP1B may function as an antagonist of the oncoprotein PTK in vivo. The Vmax, Km and Kcat of 37 kDa PTP1B mutants toward RCML are shown in Figure 2.

PTP1B and the EGF Receptor

Expression of PTP1B D181A in COS cells leads to enhanced phosphorylation of tyrosyl residues in a 180 kDa protein and in proteins of 120 and 70 kDa. When a GST-PTP1B D181A fusion protein is expressed in COS cells and precipitated on Glutathione-Sepharose, the 180 kDa, and smaller quantities of p120 and p70, were coprecipitated. The p180 protein was identified as the epidermal growth factor (EGF) receptor by immunoblotting. The identity of

the p120 and p70 proteins is unclear; however, the latter is not src, p62 or paxillin.

Expression of PTP1B D181A in COS cells induces tyrosine phosphorylation of the EGF receptor in the absence of its ligand, EGF, indicating that the mutant PTP is exerting its effects in the intact cell and not post-lysis. The equivalent D199A PTP-PEST mutant does not interact with the EGF receptor, indicating the specificity of this substrate interaction.

Autophosphorylation of the EGF receptor is required for the interaction with PTP1B D181A. Mutants of the receptor that are either kinase-dead or in which the autophosphorylation sites have been deleted do not interact with PTP1B D181A. In v-src-expressing cells, a plethora of tyrosine phosphorylated proteins were observed, but phosphorylation of the EGF receptor was not detected. Under these conditions, PTP1B D181A bound predominantly to a 70 kDa tyrosine phosphorylated protein.

As a result of this work, it appears that PTP1B can modulate EGF-induced signalling pathways, perhaps including the pathways of many diseases, including breast cancer.

Preferential Dephosphorylation of a 130 kDa Phosphotyrosine-Containing Protein by PTP-PEST

In order to investigate the substrate specificity of PTP-PEST in vitro, aliquots of pervanadate-treated HeLa cell lysates were incubated on ice, yielding 50-100 distinct phosphotyrosine-containing proteins as judged by immunoblotting of the cell lysate using the monoclonal anti-phosphotyrosine antibody G104. Purified full-length PTP-PEST (expressed in Sf9 cells using recombinant baculovirus), PTP-PEST catalytic domain, or PTP1B catalytic domain (37 kDa form) was then added to the lysate, and aliquots were removed at various time points for analysis by SDS-PAGE followed by anti-phosphotyrosine immunoblotting.

Surprisingly, a prominent 130 kDa phosphotyrosine band (p130) was selectively dephosphorylated by PTP-PEST within 10 minutes, whereas the intensity of all the other bands was essentially unchanged even after 60 minutes of incubation with PTP-PEST. Long incubations with higher concentrations of PTP-PEST (greater than 100-fold) resulted in the complete removal of all phosphotyrosine bands from the lysate. However, under all conditions tested, p130 was found to be dephosphorylated more rapidly than all other bands present.

The selective dephosphorylation of p130 by PTP-PEST was also observed using a truncated form of the phosphatase (amino acid residues 1-305) which essentially contains only the catalytic domain of the enzyme. This result suggests that the striking substrate preference displayed by PTP-PEST in this analysis is an inherent property of the phosphatase catalytic domain, whereas the C-terminal 500 amino acid residues have little discernible effect on the substrate specificity of the enzyme.

The specificity of the interaction between PTP-PEST and p130 was addressed using the catalytic domain of PTP1B (amino acid residues 1-321) in dephosphorylation reactions. When added at a similar molar concentration to that used for PTP-PEST, PTP1B was found to dephosphorylate fully and rapidly (within 15 minutes) most of the phosphotyrosine-containing proteins present in the pervanadate-treated HeLa lysate. In addition, the time course of dephosphorylation of p130 was not significantly more rapid than that of the other phosphotyrosine bands dephosphorylated by PTP1B. It should be noted, however, that these <u>in vitro</u> dephosphorylation results are not truly illustrative of the substrate specificity of PTP1B <u>in vivo</u> for several reasons. First, only the isolated catalytic subunit was used in this particular experiment. Furthermore, <u>in vivo</u> substrate specificity may be quite different due to the intracellular distribution of both the PTP and potential substrates. That

is, in vitro dephosphorylation experiemnts may utilize substrates which the PTP is capable of dephosphorylating but which it would not have access to in vivo. The phenomenon of differing substrate specificity depending upon different physiologic contexts is illustrated by a comparison of this data with the in vivo PTP1B work described above, wherein PTP1B showed specificity for only three proteins.

Identification of Phosphotyrosine-Containing p130 Protein as $p130^{cas}$ by Substrate Trapping

Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography and aliquots of the fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-$p130^{cas}$ antibodies. Aliquots of all samples analyzed were then incubated with an affinity matrix containing a substrate trapping PTP-PEST mutant, comprising full length PTP-PEST in which Asp199 is changed to alanine (D199A), bound to covalently coupled protein A-Sepharose/antibody (AG25) beads. Proteins associated with PTP-PEST were then analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-$p130^{cas}$ antibodies.

Anti-phosphotyrosine immunoblotting of the column fractions showed that the p130 phosphotyrosine band eluted as a single peak in fractions 11-14 (approx. 0.3 M NaCl). In view of the abundance of tyrosine phosphorylated p130 in HeLa lysates, it appeared likely that p130 represents a previously identified phosphotyrosine-containing 130 kDa protein. Several potential candidates were identified in the literature, including the focal adhesion kinase $p125^{FAK}$, ras-GAP, gp130 and $p130^{cas}$. Of these candidates, $p130^{cas}$ has been identified as a particularly prominent phosphotyrosine band in a wide variety of systems, including v-crk (Mayer and Hanafusa, Proc. Natl. Acad. Sci. USA 87: 2638-2642 (1990); Mayer et al., Nature 332:272-275 (1988))

and src (Kanner et al., Proc. Natl. Acad. Sci. USA 87:3328-3332 (1990); Reynolds et al., Mol. Cell. Biol. 9: 3951-3958 (1989)) transformed fibroblasts, integrin-mediated cell adhesion (Nojima et al., J. Biol. Chem. 270:15398-15402 (1995); Petch et al., J. Cell Science 108:1371-1379 (1995); Vuori and Ruoslahti, J. Biol. Chem. 270:22259-22262 (1995)) and PDGF stimulated 3T3 cells (Rankin and Rozengurt, J. Biol. Chem. 269:704-710 (1994)).

Therefore, the possibility that the p130 phosphotyrosine band corresponds to $p130^{cas}$ was tested by immunoblotting the Mono Q fractions using an antibody to $p130^{cas}$. The 130 kDa band corresponding to $p130^{cas}$ eluted in the same fractions as the p130 tyrosine phosphorylated band, and displayed a similar apparent molecular weight, suggesting that they might represent the same protein. Furthermore, $p130^{cas}$ immunoprecipitated from these fractions was found to be phosphorylated on tyrosyl residues.

A mutant form of PTP-PEST (D199A) was generated by site-directed mutagenesis, and the mutant enzyme was purified following expression using recombinant baculovirus. When assayed using tyrosine phosphorylated RCM-Lysozyme as substrate, the purified mutant enzyme exhibited a specific activity which was approximately 10,000 fold lower than that of the wild type enzyme (Garton and Tonks, unpublished data). This purified protein was bound to an affinity matrix comprised of an anti-PTP-PEST monoclonal antibody (AG25) covalently coupled to Protein A-Sepharose beads, then incubated with each of the Mono Q fractions. After 45 minutes of incubation, proteins associating with the mutant PTP-PEST were collected by centrifugation, the beads were washed, and SDS-PAGE sample buffer was added. Associated proteins were then analyzed by immunoblotting using the monoclonal anti-phosphotyrosine antibody G104.

The mutant PTP-PEST protein was found to associate with a single phosphotyrosine-containing protein, the molecular weight (130 kDa) and Mono Q elution position (fractions 11-14) of which coincided with those of $p130^{cas}$. Immunoblotting of the PTP-PEST-associated proteins using the $p130^{cas}$ antibody demonstrated that the 130 kDa tyrosine phosphorylated protein trapped by the mutant PTP-PEST is indeed $p130^{cas}$. These data further support the hypothesis that $p130^{cas}$ is a potential physiologically relevant substrate for PTP-PEST.

Determination of Structural Features of PTP-PEST Involved in Specific Interaction with Tyrosine Phosphorylated $p130^{cas}$

The interaction between $p130^{cas}$ and PTP-PEST was investigated further in substrate trapping experiments using various purified mutant forms of PTP-PEST to precipitate proteins from pervanadate-treated HeLa lysates. Several affinity matrices were incubated with pervanadate-treated HeLa cell lysate, and proteins associated with the beads were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-$p130^{cas}$ antibodies.

The wild type full-length phosphatase was found to be incapable of stable association with tyrosine phosphorylated $p130^{cas}$, whereas both the PTP-PEST (D199A) mutant protein and a mutant lacking the active site cysteine residue (C231S) specifically precipitated $p130^{cas}$ from the lysate. The inability of the wild type phosphatase to precipitate tyrosine phosphorylated $p130^{cas}$ presumably reflects the transient nature of the normal interaction between PTP-PEST and tyrosine phosphorylated $p130^{cas}$, which is likely to be concluded as soon as $p130^{cas}$ is dephosphorylated by PTP-PEST.

Since the C-terminal 500 amino acids of PTP-PEST contain several proline-rich regions which resemble src homology-3 (SH3) domain binding sequences, it appeared

plausible that the specificity of the interaction between PTP-PEST and $p130^{cas}$ might depend to some extent on association of these segments with the SH3 domain of $p130^{cas}$. The possible contribution of the C-terminal segment of PTP-PEST in the observed specific interaction of PTP-PEST with $p130^{cas}$ was therefore addressed in further substrate trapping experiments using GST fusion proteins containing the catalytic domain of PTP-PEST alone, in both wild type and mutant (D199A) forms. The mutant catalytic domain of PTP-PEST fused to GST was found to precipitate the $p130^{cas}$ phosphotyrosine band specifically, whereas both the wild type fusion protein and GST alone failed to precipitate $p130^{cas}$. The specific interaction between PTP-PEST and $p130^{cas}$ observed in these experiments therefore appears to be an intrinsic property of the catalytic domain of PTP-PEST, emulating the observed preference of the active PTP-PEST catalytic domain for dephosphorylation of $p130^{cas}$ in vitro.

Specificity of Interaction Between Mutant PTP-PEST and Tyrosine Phosphorylated $p130^{cas}$

In view of the relative abundance of tyrosine phosphorylated $p130^{cas}$ in the pervanadate-treated HeLa cell lysate, the possibility that the observed selective binding of PTP-PEST inactive mutant proteins to $p130^{cas}$ was substrate-directed (reflecting the abundance of this potential substrate relative to the other phosphotyrosine-containing proteins present in the lysate) rather than enzyme-directed (reflecting a genuine substrate preference of PTP-PEST) was considered; this possibility was addressed in two ways. First, inactive mutant forms of the catalytic domain of PTP1B were used to trap potential substrates for this enzyme from the pervanadate-treated HeLa lysates. Again it was found that the wild type phosphatase was incapable of stable interaction with any phosphotyrosine-

containing protein, whereas mutant variants of the PTP1B phosphatase domain (comprising Cys or Asp mutations analogous to those described above for PTP-PEST) associated with many tyrosine phosphorylated proteins. This was especially apparent for the aspartic acid mutant of PTP1B (D181A), which appeared to precipitate essentially all phosphotyrosine-containing proteins from the lysate with similar efficacy. These data emphasize the specific nature of the interaction between PTP-PEST and $p130^{cas}$, which appears to be a property peculiar to the PTP-PEST catalytic domain, rather than a feature shared by all PTP catalytic domains.

The specificity of the interaction between PTP-PEST and $p130^{cas}$ was addressed further following pervanadate-treatment of several different cell lines (Wi38, 293, COS, MCF10A, C2C12, MvLu), yielding a different array of tyrosine phosphorylated proteins in each case; the resultant lysates were analyzed by SDS-PAGE followed by anti-phosphotyrosine immunoblotting. Aliquots were incubated with PTP-PEST (D199A) affinity matrix or control matrix, and tyrosine phosphorylated proteins associating with PTP-PEST were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine or anti-$p130^{cas}$ antibodies as described above.

In each case, the D199A mutant PTP-PEST protein precipitated a single broad phosphotyrosine band with an apparent molecular weight between 120 and 150 kDa in different cell lines, whereas the affinity matrix alone failed to precipitate any phosphotyrosine-containing protein. Immunoblotting of the precipitates with a $p130^{cas}$ antibody revealed that the protein precipitated from all cell lysates corresponded to $p130^{cas}$; the observed molecular weight variation between different cell lines presumably reflects either species differences in the molecular weight of $p130^{cas}$ or expression of different

alternatively spliced forms (Sakai et al., EMBO J. 13:3748-3756 (1994)).

The relative abundance of tyrosine phosphorylated $p130^{cas}$ in the PTP-PEST precipitates appeared to correlate approximately with the abundance of $p130^{cas}$ protein in the lysates (data not shown). Surprisingly, regardless of the abundance of tyrosine phosphorylated $p130^{cas}$ in the lysates, $p130^{cas}$ was invariably the only phosphotyrosine-containing protein in the precipitates, even in 293 cell lysates which contained very little $p130^{cas}$ protein but which displayed a wide variety of other abundantly tyrosine phosphorylated proteins. Similarly, when lysates of pervanadate-treated 293 cells (containing tyrosine phosphorylated $p130^{cas}$ in amounts which are undetectable by anti-phosphotyrosine immunoblotting of the lysate) were incubated with active PTP-PEST, no visible dephosphorylation of any phosphotyrosine band occurred (Garton and Tonks, unpublished data). These results indicate that the affinity of PTP-PEST for $p130^{cas}$ is substantially greater than for any other substrate present, and further emphasizes the remarkable substrate selectivity of PTP-PEST for $p130^{cas}$.

Vanadate Inhibition of Tyrosine Phosphorylated $p130^{cas}$ Association with Mutant PTP-PEST

A consistent observation of this work was that, in contrast to the inactive mutant PTP-PEST, the wild type enzyme failed to associate in a stable complex with tyrosine phosphorylated $p130^{cas}$, suggesting that the observed association is active site-directed. In order to investigate this possibility, mutant PTP-PEST (D199A) was incubated with the PTP inhibitor vanadate at various concentrations prior to addition of pervanadate-treated HeLa cell lysate. The extent of association of $p130^{cas}$ with PTP-PEST was then analyzed. PTP-PEST affinity matrix, comprising full length PTP-PEST (D199A) bound to covalently

coupled protein A-Sepharose/antibody (AG25) beads, was incubated for 10 minutes on ice in the presence of varying concentrations of sodium orthovanadate. The samples were then incubated with aliquots of pervanadate-treated HeLa cell lysate; associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine or anti-$p130^{cas}$ antibodies. The activity of wild type PTP-PEST was also determined under the same conditions, using tyrosine phosphorylated ^{32}P-labelled RCM-lysozyme as substrate.

The association was found to be potently disrupted by vanadate, with a concentration-dependence similar to that of vanadate inhibition of wild type PTP-PEST, and complete disruption being observed at 10 mM vanadate. Since PTP inhibition by vanadate presumably results from a direct interaction of vanadate with the active site cysteine residue of the enzyme (Denu et al., Proc. Natl. Acad. Sci. USA 93:2493-2498 (1996)), this result supports the hypothesis that the stable association of mutant PTP-PEST with tyrosine phosphorylated $p130^{cas}$ is mediated by direct interactions between active site residues within PTP-PEST, in particular the active site cysteine residue, and phosphotyrosine moieties within $p130^{cas}$.

Association of Endogenous $p130^{cas}$ with Transfected Mutant PTP-PEST in COS Cells

The work described above strongly suggests that $p130^{cas}$ represents a potential physiologically significant substrate for PTP-PEST. In order to assess whether PTP-PEST interacts with $p130^{cas}$ in intact cells, COS cells were transfected with plasmids encoding wild type or mutant forms of PTP-PEST (D199A or C215S). The cells were treated with pervanadate 30 minutes prior to lysis, PTP-PEST proteins were immunoprecipitated, and associated tyrosine phosphorylated proteins were analyzed by anti-phosphotyrosine immunoblotting of the resultant

precipitates. Lysates were also incubated with covalently coupled protein A-Sepharose/anti-PTP-PEST (AG25) beads and associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody.

Under these conditions, the phosphotyrosine-containing band corresponding to $p130^{cas}$ was again unique in its ability to associate with the C231S PTP-PEST protein, indicating that $p130^{cas}$ can be specifically selected by PTP-PEST as a substrate in an intracellular context in the presence of a large number of alternative possible substrates. Neither the wild type nor the D199A form of PTP-PEST was capable of a stable interaction with tyrosine phosphorylated $p130^{cas}$ in pervanadate-treated COS cells.

The binding of both wild type and D199A PTP-PEST to tyrosine phosphorylated $p130^{cas}$ under these conditions is most likely prohibited by the presence of pervanadate bound to the active site cysteine residue of PTP-PEST (Denu et al., Proc. Natl. Acad. Sci. USA 93:2493-2498 (1996)), which effectively excludes the binding of phosphotyrosine residues of $p130^{cas}$. The ability of the C231S mutant PTP-PEST to associate in a stable complex with $p130^{cas}$ in the presence of pervanadate suggests that this mutant protein is largely unaffected by pervanadate, indicating that the normal mode of inhibition of PTPs by vanadate ions depends critically on direct interactions between vanadate and the thiolate anion of the PTP active-site cysteine residue. These observations therefore lend further support to the existence of an exclusive interaction between PTP-PEST and $p130^{cas}$ which appears to be entirely active site-directed, and therefore reflects the genuine, inherent, highly restricted substrate preference of PTP-PEST for $p130^{cas}$.

Results described herein implicate $p130^{cas}$ as a physiologically relevant substrate for PTP-PEST. Furthermore, the observed stringency and exclusivity of the interaction between PTP-PEST and $p130^{cas}$ in a wide variety

of cell lines suggest that $p130^{cas}$ may be a unique high affinity substrate for PTP-PEST, although the possibility that other significant PTP-PEST substrates may exist cannot be excluded at present. In particular, it is unclear whether pervanadate-treated cells display a complete spectrum of all possible tyrosine phosphorylated proteins; in fact this appears unlikely since pervanadate treatment presumably results only in an increase in tyrosine phosphorylation of proteins which are to some extent constitutively phosphorylated, but which are normally rapidly dephosphorylated, within the cell. Potential substrates lacking from pervanadate-treated cells therefore presumably include substrates of protein tyrosine kinases (PTKs) which are normally present in an inactive state, such as ligand-stimulated receptor PTKs, and the recently described calcium regulated kinase PYK2 (Lev et al., Nature 376: 737-745 (1995)). Regardless of these considerations, the ability of PTP-PEST to select $p130^{cas}$ exclusively as a substrate from lysates of several different cell lines, containing a combined total of at least one hundred different potential substrates (many of which presumably contain multiple sites of phosphorylation), clearly demonstrates that the substrate specificity of PTP-PEST is highly restricted.

Many intracellular PTPs are limited in their substrate availability due to strict confinement within a particular subcellular location; examples include PTP1B, which is localized to the cytoplasmic face of the endoplasmic reticulum (Frangioni et al., Cell 68:545-560 (1992)), and TCPTP which is either nuclear (Tillmann et al., Mol. Cell. Biol. 14:3030-3040 (1994)) or localized to the endoplasmic reticulum, depending upon which alternative spliced form is expressed (Lorenzen et al., J. Cell Biol. 131:631-643 (1995)). Alternatively, certain PTPs appear to be highly regulated, requiring activation before appreciable activity

can be demonstrated. For example, the SH2 domain-containing PTPs, SHP1 and SHP2, display relatively low activity in vitro, but can be considerably activated by several mechanisms including C-terminal truncation (Zhao et al., J. Biol. Chem. 268:2816-2820 (1993)), addition of certain phospholipids (Zhao et al., Proc. Natl. Acad. Sci. USA 90:4251-4255 (1993)), or SH2 domain-mediated binding of appropriate phosphotyrosine-containing peptides (Lechleider et al., J. Biol. Chem. 268:21478-21481 (1993)).

However, PTP-PEST exhibits high specific activity in vitro (35,000 U/mg), and is a predominantly (90-95%) soluble PTP within cells (Garton and Tonks, unpublished data); in principle, therefore, it may act potently on any substrate accessible to the cytoplasm. This accessibility may partly underlie the necessity for PTP-PEST to possess an inherently constrained substrate specificity. The demonstration that mutant PTP-PEST is capable of exclusively associating with $p130^{cas}$ in an intracellular context in the presence of many other tyrosine phosphorylated proteins, is an indication that the narrow substrate specificity of the enzyme may result in PTP-PEST having a negligible influence on the phosphorylation state of the majority of tyrosine phosphorylated proteins within the cell, even though those substrates are largely accessible to PTP-PEST.

The role of $p130^{cas}$ in cellular transformation by the v-crk and v-src oncogenes is unclear, although there is a general correlation between the level of tyrosine phosphorylation of $p130^{cas}$ and the degree of transformation in cells expressing different forms of crk or src (Kanner et al., EMBO J. 10:1689-1698 (1991); Mayer and Hanafusa, J. Virol. 64:3581-3589 (1990)). Furthermore, enhanced tyrosine phosphorylation of $p130^{cas}$ has also been observed in cells transformed by c-Ha-ras and by ornithine decarboxylase overexpression (Auvinen et al., Mol. Cell. Biol. 15:6513-6525 (1995)). Expression of antisense cDNA encoding

p130^{cas} in these cells results in a partial reversion of the transformed phenotype. These observations suggest that aberrant tyrosine phosphorylation of p130^{cas} is a common feature of cells transformed by several disparate mechanisms and that p130^{cas} may be required for full manifestation of the transformed state. Dephosphorylation of p130^{cas} by PTP-PEST is therefore a potentially important regulatory mechanism for counteracting the transforming effects of various oncogenes.

Tyrosine phosphorylation of p130^{cas} has been observed in fibroblasts following integrin-mediated cell adhesion to extracellular matrix proteins (Nojima et al., J. Biol. Chem. 270:15398-15402 (1995); Petch et al., J. Cell Science 108:1371-1379 (1995); Vuori and Ruoslahti, J. Biol. Chem. 270:22259-22262 (1995)). Under these conditions, using an antibody (4F4) that predominantly recognizes tyrosine phosphorylated p130^{cas} (Kanner et al., EMBO J. 10:1689-1698 (1991); Petch et al., J. Cell Science 108:1371-1379 (1995)) it was shown that phosphorylated p130^{cas} is localized to focal adhesions (Petch et al., J. Cell Science 108:1371-1379 (1995)), whereas fractionation studies have demonstrated that the normal cellular location of the majority of non-phosphorylated p130^{cas} is the cytosol (Sakai et al., EMBO J. 13:3748-3756 (1994)). Furthermore, in crk-transformed fibroblasts, tyrosine phosphorylated p130^{cas} is detected only in insoluble fractions (Sakai et al., EMBO J. 13:3748-3756 (1994)), suggesting that both cell adhesion- and transformation-mediated phosphorylation of p130^{cas} is associated with redistribution of the protein from the cytosol to focal adhesions.

It is plausible that the redistribution of tyrosine phosphorylated p130^{cas} may be driven by its association with FAK, which is constitutively associated with focal adhesions due to its C-terminal focal adhesion targeting domain (Hildebrand et al., J. Cell Biol. 123:993-1005

(1993); Schaller et al., Proc. Natl. Acad. Sci. USA 89:5192-5196 (1992)). The sequestration of tyrosine phosphorylated $p130^{cas}$ in focal adhesions both in transformed cells, and following integrin-mediated cell adhesion, strongly suggests a role for $p130^{cas}$ in signalling events in this region of the cell. One consequence of the redistribution of tyrosine phosphorylated $p130^{cas}$ is likely to be that, in addition to localizing $p130^{cas}$ to a region of the cell containing abundant protein tyrosine kinase activity, the phosphorylated protein will be relatively inaccessible to the cytosolic phosphatase PTP-PEST. This raises the possibility that the role of PTP-PEST in dephosphorylating $p130^{cas}$ may be to prevent inappropriate tyrosine phosphorylation of the cytosolic pool of $p130^{cas}$, thus preventing formation of signalling complexes assembled around tyrosine phosphorylated $p130^{cas}$ in inappropriate cellular locations.

Several mitogenic factors potently stimulate tyrosine phosphorylation of $p130^{cas}$. These include agents acting through heterotrimeric G protein-coupled receptors such as lysophosphatidic acid (Seufferlein and Rozengurt, J. Biol. Chem. 269:9345-9351 (1994)), bradykinin (Leeb-Lundberg et al., J. Biol. Chem. 269: 24328-24344 (1994)), and bombesin (Zachary et al., J. Biol. Chem. 267:19031-19034 (1992)), as well as growth factors that activate receptor tyrosine kinases, namely PDGF (Rankin and Rozengurt, J. Biol. Chem. 269:704-710 (1994)), EGF and NGF (Ribon and Saltiel, J. Biol. Chem. 271:7375-7380 (1996)). These observations suggest roles for $p130^{cas}$ in regulation of mitogenic signalling pathways, presumably involving assembly of signalling complexes based on tyrosine phosphorylated $p130^{cas}$. The identities of the proteins involved in these complexes are not established, but are likely to include SH2 domain-containing adaptor proteins such as crk (Ribon and Saltiel, J. Biol. Chem. 271:7375-7380 (1996)), and its

associated proteins (Feller et al., Oncogene 10:1465-1473 (1995); Hasegawa et al., Mol. Cell. Biol. 16:1770-1776 (1996); Knudsen et al., J. Biol. Chem. 269:32781-32787 (1994); Matsuda et al., Mol. Cell. Biol. 14: 5495-5500 (1994); Tanaka et al., Proc. Natl. Acad. Sci. USA 91:3443-3447 (1994)). Therefore tyrosine phosphorylation and dephosphorylation of $p130^{cas}$ potentially plays a central role in regulating the formation of such complexes, thereby influencing downstream events in mitogenic signalling.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

CLAIMS

We claim:

1. A protein tyrosine phosphatase wherein the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which:
   a) does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; or
   b) is selected from the group consisting of: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, glutamine, lysine, arginine and histidine, and wherein the phosphatase binds to a tyrosine phosphorylated substrate and is catalytically attenuated.

2. A protein tyrosine phosphatase according to claim 1(a) which is selected from the group consisting of: PTP1B (and wherein for example the invariant aspartate residue is located at position 181), PTP-PEST (and wherein for example the invariant aspartate residue is located at position 199), PTPγ,MKP-1, DEP-1, PTPμ,PTPX1, PTPX10 and PTPH1.

3. A protein tyrosine phosphatase according to claim 1(b) which is a PTP-PEST phosphatase in which the amino acid at position 231 is replaced with a serine residue.

4. A method of identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase, comprising the steps of:
   a) combining at least one tyrosine phosphorylated protein with at least one protein tyrosine

phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, under conditions appropriate for formation of a complex between the tyrosine phosphorylated protein and the protein tyrosine phosphatase, thereby producing a combination; and

b) determining the presence of absence of a complex in the combination

wherein the presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with which it forms a complex.

5. A method according to claim 4, wherein the protein tyrosine phosphatase is as defined in any one of claims 1-3.

6. A method according to claim 4 or claim 5 wherein the tyrosine phosphorylated protein is selected from the group consisting of: $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor.

7. A kit for identifying a tyrosine phosphorylated protein substrate of a protein tyrosine phosphatase comprising:

a) at least one protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

b) ancillary reagents suitable for use in detecting the presence or absence of a complex between the protein tyrosine phosphatase and a tyrosine phosphorylated protein,

wherein for example the protein tyrosine phosphatase is as defined in any one of claims 1-3.

8. A method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphatase, comprising the steps of:

a) identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase;

b) combining the tyrosine phosphorylated protein and the protein tyrosine phosphatase and an agent to be tested under conditions suitable for interaction between the tyrosine phosphorylated protein and the protein tyrosine phosphatase, thereby forming a combination;

c) determining the amount of enzymatic activity in the combination; and

d) comparing the amount of enzymatic activity determined in (c) with the amount of enzymatic activity in the absence of the agent to be tested, under conditions suitable for interaction between the tyrosine phosphorylated protein and the protein tyrosine phosphatase,

wherein a difference in the enzymatic activity indicates that the agent alters the interaction between the protein tyrosine phosphatase and the tyrosine phosphorylated protein.

9. A method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate

of the protein tyrosine phosphatase, comprising the steps of:

a) identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase;

b) combining the tyrosine phosphorylated protein, a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and an agent to be tested, under conditions suitable for interaction between the tyrosine phosphorylated protein and the protein tyrosine phosphatase, thereby forming a combination;

c) determining the extent of binding between the tyrosine phosphorylated protein and the protein tyrosine phosphatase in the combination; and

d) comparing the extent of binding determined in (c) with the extent of binding in the absence of the agent to be tested, under conditions suitable for interaction between the tyrosine phosphorylated protein and the protein tyrosine phosphatase,

wherein a difference in the extent of binding indicates that the agent alters the interaction between the protein tyrosine phosphatase and the tyrosine phosphatase and the tyrosine phosphorylated protein.

10. A method according to claim 8 or claim 9 wherein if the amount of enzymatic activity or the extent of binding, respectively, is:

a) greater in the presence of the agent to be tested than in the absence of the agent, then the agent

enhances the interaction between the protein tyrosine phosphatase and the tyrosine phosphorylated protein; or

b) less in the presence of the agent to be tested than in the absence of the agent, then the agent inhibits the interaction between the protein tyrosine phosphatase and the tyrosine phosphorylated protein.

11. A protein tyrosine phosphatase (e.g. as defined in any one of claims 1-3) for use in therapy, prophylaxis or diagnosis, for example in:

a) the treatment of conditions in which a reduction in the activity of a tyrosine phosphorylated protein is indicted; and/or

b) reducing the activity of a tyrosine phosphorylated protein; and/or

c) a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby formation of the complex reduces the activity of the tyrosine phosphorylated protein; and/or

d) the treatment of conditions in which a reduction in the transforming effects of oncogenes associated with $p130^{cas}$ phosphorylation is indicated; and/or

e) reducing the transforming effects of oncogenes associated with $p130^{cas}$ phosphorylation; and/or

f) a method of reducing the transforming effects of oncogenes associated with $p130^{cas}$ phosphorylation

comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates $p130^{cas}$ and reducing the transforming effects of oncogenes associated with $p130^{cas}$ phosphorylation; and/or

g) the treatment, therapy, diagnosis or prophylaxis of cancer (for example cancers associated with $p130^{cas}$ phosphorylation); and/or

h) the treatment of conditions associated with oncogneic activity (e.g. with v-crk, v-src and/or c-Ha-ras activity); and/or

i) the treatment of conditions in which a reduction in the formation of signalling complexes associated with $p130^{cas}$ is indicted; and/or

j) reducing the formation of signalling complexes associated with $p130^{cas}$; and/or

k) a method of reducing the formation of signalling complexes associated with $p130^{cas}$ comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates $p130^{cas}$ and reducing the formation of signalling complexes associated with $p130^{cas}$, and/or

l) the prevention of the induction of mitogenic pathways;

m) the treatment of conditions in which the prevention of the induction of mitogenic pathways is indicated;

n) the treatment of conditions in which a reduction in the cytotoxic effects associated with protein tyrosine phosphatase administration or over-expression is indicated; and/or

o) reducing the cytotoxic effects associated with protein tyrosine phosphatase administration or over-expression; and/or

p) a method for reducing the cytotoxic effects associated with protein tyrosine phosphatase administration or over-expression comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, in place of a corresponding wild type protein tyrosine phosphatase; and/or

q) in a method according to any one of claims 13-20.

12. Use of a protein tyrosine phosphatase for the manufacture of a medicament for treatment, prophylaxis or diagnosis (for example for use in the treatments defined in claim 11).

13. A method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant

alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby formation of the complex reduces the activity of the tyrosine phosphorylated protein.

14. A method according to claim 13, wherein the tyrosine phosphorylated protein is selected from the group consisting of: $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor.

15. a method according to claim 13, wherein the protein tyrosine phosphatase is selected from the group consisting of: PTP1B, PTP-PEST, PTP_7, MKP-1, DEP-1, PTP_7, PTPX1, PTPX10 AND PTPH1.

16. A method of reducing the transforming effects of oncogenes associated with $p130^{cas}$ phosphorylation comprising of administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p 130^{cas}, thereby negatively regulating the downstream effects of $p130^{cas}$ and reducing the transforming effects of oncogenes associated with $p130^{cas}$ phosphorylation.

17. A method according to claim 16, wherein the oncogene is selected from the group consisting of: v-crk, v-src and c-Ha-ras.

18. A method of reducing formation of signalling complexes associated with $p130^{cas}$ comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST OR PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates $p130^{cas}$, thereby negatively regulating the downstream effects of $p130^{cas}$ and reducing the formation of signalling complexes associated with $p130^{cas}$.

19. A method according to claim 18, which prevents the induction of mitogenic pathways.

20. A method of reducing cytotoxic effects associated with protein tyrosine phosphatase administration or over expression, comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, in place of a corresponding wild type protein tyrosine phosphatase.

70 80 90 100 110 120 130
Hum_PTP1B DyINASLI KMEEAQRSYILTqGPLPNTCGHFWEMVWE QKSRGVVMLNRVM
Hum_TCPTP DyINASLV DIEEAQRSYILTqGPLPNTCCHFWLMVWQ QKTKAVVMLNRIV
Hum_PTP_xi_D1 DGKLTDyINANYV DGYN RPKAYIAAqGPLKSTAEDFWRMIWE HNVEVIVMITNLV
Hum_PTP_zeta_D1 DGKLTDyINANYV DGYN RPKAYIAAqGPLKSTFEDFWRMIWE HNVEVIVMITNLV
Hum_PTP_gamma_D DSKHSDyINANYV DGYN KAKAYIATqGPLKSTFEDFWRMIWE QNTGIIVMITNLV
Dros_PTP99A_D1 KKNL DyINANFI DGYQ KGHAFIGTqGPLPDTFDCFWRMIWE QRVAIIVMITNLV
Hum_LCA_D1 PGS DyINANYI DGYR KQNAYIATqGPLPETMGDFWRMVWE QRTATVVMMTRLE
Hum_PTP_mu_D1 TNS DyINGNYI DGYH RPNHYIATqGPMQETIYDFWRMVWH ENTASIIMVTNLV
Hum_PTP_alpha_D1 PDS DyINASFI NGYQ EKNKFIAAqGPKEETVNDFWRMIWE QNTATIVMVTNLK
Hum_PTP_opsilon_D PCS DyINASYI DGYK EKNKFIAAqGPKQETVNDFWRMVWE QKSATIVMLTNLK
Mouso_CD45_D1 AGS TyINASYI DGFK EPRKYIAAqGPRDETVDDFWRMIWE QKATVIVMVTRCE
Hum_SH.PTP2 EPV SDyINANIIMPEFETKCNNSK PKKSYIATqGCLQNTVNDFWRMVFQ ENSRVIVMTTKEV
Hum_SH.PTP1 IPG SDyINANYIKNQLL GPDE NAKTYIASqGCLEATVNDFWQMAWQ ENSRVIVMTTREV
Hum_PTP_bola PCS DyINASYI PGNN FRREYIVTqGPLPGTKDDFWKMVWE QNVHNIVMVTQCV
Dros_PTP10D EGS DyINANYV PGHN SPREFIVTqGPLHSTRDDFWRMCWE SNSRAIVMLTRCF
Hum_SAP.1 PGS DyINASFM PGLW SPQEFIATqGPLPQTVGDFWRLVWE QQSHTLVMLTNCM
Ral_PTP_STEP DPL SSyINANYI RGYNGEEKVYIATqGPIVSTVVDFWRMVWQ ERTPIIVMITNIE
Dros_PTP69A_D1 QTT DyINANFV IGYK ERKKFICAqGPMESTIDDFWRMIWE QHLEIIVILTNLE
Hum_MEG2 TQT DyINASFM DGYK QKNAYIGTqGPLENTYRDFWLMVWE QKVLVIVMTTRFE
Hum_PTP.PEST QDS DyINANFI KGVY GPKAYVATqGPLANTVIDFWRMVWE YNVVIIVMACREF
Hum_PTPH1 INA SyVNMEI PAAN LVNKYIATqGPLPHTCAQFWQVVWD QKLSLIVMLTTLT
Dici_PTP1 EGS DyINANYI DGAY PKQFICTqGPLPNTIADFWRMVWE NRCRIIVMLSRES
Fiss_yeast_pyp1 EL DyINASFI KTETSNYIACqGSISRSISDFWHMVWDNVENIGTIVMLGSLF
Fiss_yeast_pyp2 S DyINASHI DVGNKKYIACqAPKPGTLLDFWEMVWHNSGTNGVIVMLTNLV
Hum_PTP_xi_D2 DGKLTDyINANYV DGYN RPKAYIAAqGPLKSTAEDFWRMIWE HNVEVIVMITNLV
Hum_LCA_D2 EGS DyINASFL DGYR QQKAYIATqGPLAESTEDFWRMLWE HNSTIIVMLTKLR
Hum_PTP_alpha_D2 ENT DyVNASFI DGYR QKDSYIASqGPLLHTIEDFWRMIWE WKSCSIVMLTELE
Hum_PTP_opsilon_D2 EYT DyINASFI DGYR QKDYFIATqGPLAHTVEDFWRMIWE WKSHTIVMLTEVQ
Hum_PTP_mu_D2 SS NyINAALM DSYK QPSAFIVTqHPLPNTVKDFWRLVLD YHCTSVVMLNDVD
Mouse_CD45_D2 SEETSKyINASFV MSYW KPEMMIAAqGPLKETIGDFWQMIFQ RKVKVIVMLTELV
Dros_PTP69A_D2 ENS TyINASFI EGYD NSETFIIAqDPFENTIGDFWRMISE QSVTTLVMISEIG
Hum_PTP_zeta_D2 GT DyINASYI MGYY QSNEFIITqHPLLHTIKDFWRMIWD HNAQLVVMIPDGQ
Hum_PTP_gamma_D2 KGT DyINASYI MGYY RSNEFIITqHPLPHTTKDFWRMIWD HNAQIIVMLPDNQ
Dros_PTP99A_D2 GEDGSDyINASWL HGFR RLRDFIVTqHPMAHTIKDFWQMVWD HNAQTVVLLSSLD
Yarsinia_PTP NyIQVG NTRTIACqYPLQSQLESHFRMLAE NRTPVLAVLASSS
PTP1Bseq.no. 70 80 90 100 110 120 130
PTP1B66
β1 β2 α2 β3 α3
Figure 1A

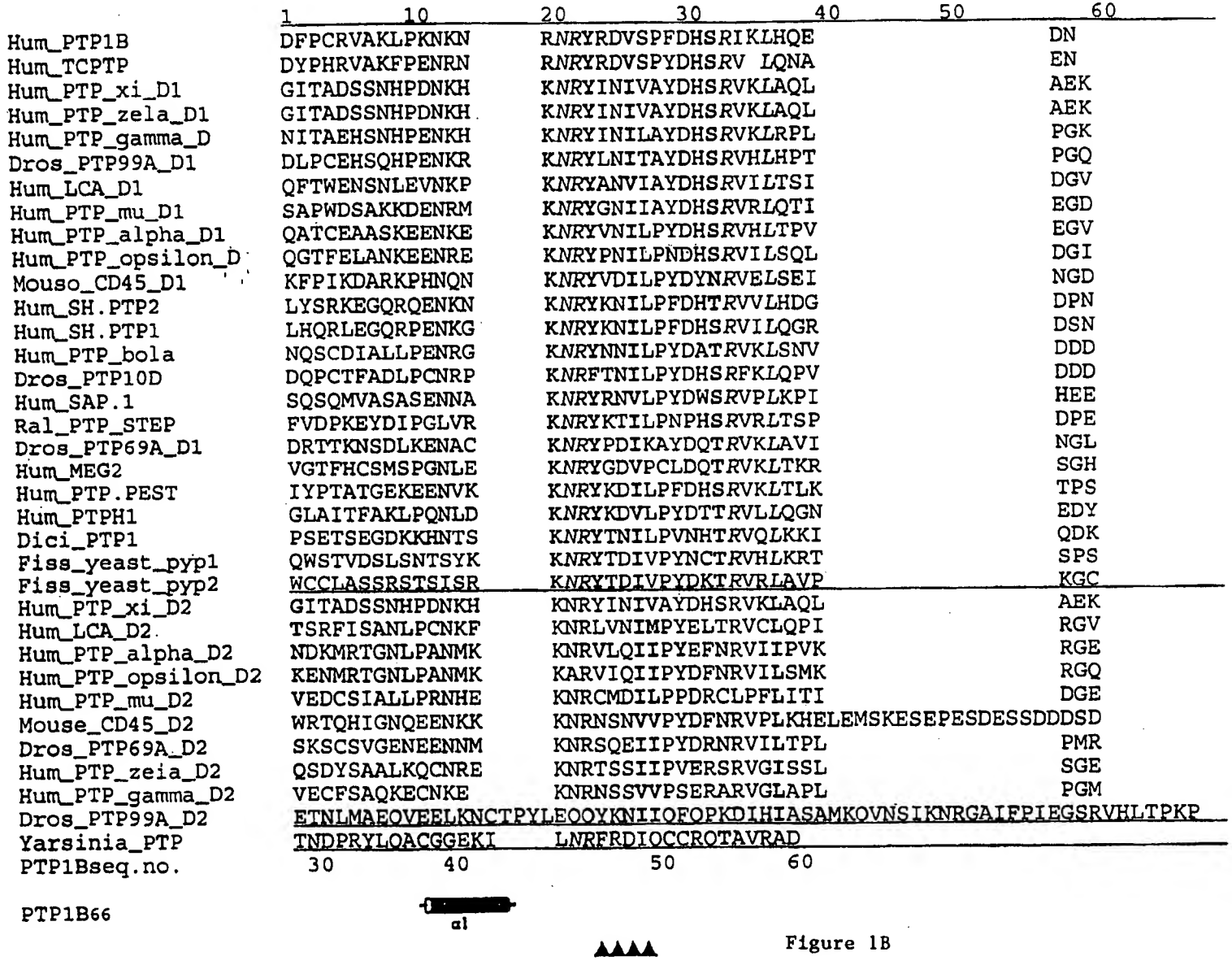

Figure 1B

	140	150	160 170 180	190 200
Hum_PTP1B	EKGSLKCA	QYWPQKEEKEM	IFEDTNLKLTLISEDIKSYYTVLELE	NLTTQETREI
Hum_TCPTP	EKESVKCA	QYWPT DDQEM	LFKETGFSVKLLSEDVKSYYTVLQLE	NINSGETRTI
Hum_PTP_xi_D1	EKGRRKCD	QYWPP ADGSE	EYGN FLVTQKSVQVLAYYTVFTLRNTKIKKG	SQKGRPSGRVV
Hum_PTP_zela_D1	EKGRRKCD	QYWP ADGSE	EYGN FLVTQKSVQVLAYYTVFTLRNTKIKKG	SQKGRPSGRVV
Hum_PTP_gamma_D	EKGRRKCD	QYWP TENSE	EYGN IIVTLKSTKIHACYTVFSIRNTKVKKGQKGNPKGRQNERVV	
Dros_PTP99A_D1	ERGRRKCD	MYWP KDGVE	TYGV IQVKLIEEEVMSTYTVLQIKHLKLKK	KKQCNTEKLV
Hum_LCA_D1	EKSRVKCD	QYWP ARGTE	TCGL IQVTLLDTVELATYTVFALH	KSGSSEKREL
Hum_PTP_mu_D1	EVGRVKCC	KYWP DDTE	IYKD IKVTLIETELLAEYVIFAVE	KRGVHEIREI
Hum_PTP_alpha_D1	ERKECKCA	QYWP DQGCW	TYGN IRVSVEDVTVLVDYTVFCIQQVG	DMTNRKPQRLI
Hum_PTP_opsilon_D	ERKEEKCH	QYWP DQGCW	TYGN IRVCVEDCVVLVDYTIFCIQPQL	PDGCKAPRLV
Mouso_CD45_D1	EGNRNKCA	EYWPSMEEGTR	AFKD IVVTINDHKRCPDYIILNVAH	KKEKATGREV
Hum_SH.PTP2	ERGKSKCV	KYWPD EYALK	EYGV MRVRNVKESAAHDYTLLKLSK	VGQGNTERTV
Hum_SH.PTP1	EKGRNKCV	PYWPE VGMQR	AYGP YSVTNCGEHDTTEYKLLQVSP	LDNGDLIREI
Hum_PTP_bola	EKGRVKCD	HYWPA DQDSL	YYGD LILQMLSESVLPEWTIFKICG	EEQLDAHRLI
Dros_PTP10D	EKGREKCD	QYWPN DTVPV	FYGD IKVQILNDSHYADWVMFMLC	RGSEQRIL
Hum_SAP.1	EAGRVKCE	HYWPL DSQPC	THGH LRVTLVGEEVMENWTVLLLL	QVEEQKTLSV
Ral_PTP_STEP	EMN EKCT	EYWP EEQV	VHDG VEITVQKVIHTEDYRLISLR	RGTEERGL
Dros_PTP69A_D1	EYNKAKCA	KYWPEKVFDTK	QFGD ILVKFAQERKTGDYIELNVSKNKAN	VGEEEDRRQI
Hum_MEG2	EGGRRKCG	QYWPLEKDSRI	RFGF LTVTNLGVENMNHYKKLEIH	NTEERQKRQV
Hum_PTP.PEST	EMGRKKCE	RYWPLYGEDPI	TFAP FKISCEDEQARTDYFILLLE	FQNESRRL
Hum_PTPH1	ERGRTKCH	QYWPD PPDVM	NHGG FHIQCQSEDCTIAYVSMLVT	NTQTGEEHTV
Dici_PTP1	ENCRIKCD	RYWPEQIGGEQFSIYGNGNEVFGTYSVELVEVIQCREIITRNIR		LTFEGETRDI
Fiss_yeast_pyp1	EAGREMCT	AYWPSNGIGDK	QVYGDYCVKQISEENVDNSRFILFEIQ	NANFPSVKKV
Fiss_yeast_pyp2	EAGSEKCS	QYWPDNKDHALCLEGG	LRISVQKYETEDLKVHLFRL	DKPNGPPKYI
Hum_PTP_xi_D2	EKGRRKCD	QYWP ADGSE	EYGN FLVTQKSVQVLAYYTVFTLRNTKIKKG	SQKGRPSGRVV
Hum_LCA_D2	EMGREKCH	QYWP AERSA	RYQY FVVDPMAEYNMPQYILFKVT	DARDGQSRTI
Hum_PTP_alpha_D2	ERGQEKCA	QYWP SDGLV	SYGD ITVELKKEEECESYTVLLVT	NTRENKSRQI
Hum_PTP_opsilon_D2	EREQDKCY	QYWP TEGSV	THGE ITIEIKNDTLSEAISIFLVTLNQPQ	ARQEEQVRVV
Hum_PTP_mu_D2	PA QLCP	QYWP ENGVH	RHGP IQVEFVSADLEEDIISFRIYNA	ARPQDGYRMV
Mouse_CD45_D2	NGDQEVCA	QYW GEGKQ	TYGD MEVEMKDTNRASAYTLFELR	HSKRKEPRTV
Dros_PTP69A_D2	D GPRKCP	RYWA DDEVQ	YDH ILVKYVHSESCPYYTFFYVT	NCKIDDTLKV
Hum_PTP_zeia_D2	NMAEDEFV	YWPNKDEPINCESFKVTLMAEEHKCLSNEEKLIIFILE		ATQDDYVLEV
Hum_PTP_gamma_D2	SLAEDEFV	YWPSREESMNCEAFTVTLISKDRLCLSNEEQIIIFILE		ATQDDYVLEV
Dros_PTP99A_D2	D INFA	QFWPDEATPIESDHY	RVKFLNKTNKSDYVSFVIQ	SIQDDYELTV
Yarsinia_PTP	EIANQRFGMPDYFR	QSGT	YGSITVESKMTQQVGLGDGINMYTLTI	REAGQKTISV
PTP1Bseq.no.	120	130	140 150 160	

PTP1B66 β5 β6 β7 β8

Figure 1C

210 220 230 240 250 260 270
Hum_PTP1B LHFHYTTwPDF G VPESPASFLNFLFKVRES GSLSPEHG PVVVHCSAgIGRSGTFC
Hum_TCPTP SHFHYTTwPDF G VPESPASFLNFLFKVRES GSLNPDHG PAVIHCSAgIGRSGTFS
Hum_PTP_xi_D1 TQYHYTQwPDM G VPEYSLPVLTFVRKAAYA KRH AVG PVVVHCSAgVGRTGTYI
Hum_PTP_zela_D1 TQYHYTQwPDM G VPEYSLPVLTFVRKAAYA KRH AVG PVVVHCSAgVGRTGTYI
Hum_PTP_gamma_D IQYHYTQwPDM G VPEYALPVLTFVRRSSAA RMP ETG PVLVHCSAgVGRTGTYI
Dros_PTP99A_D1 YQYHYTNwPDH G TPDHPLPVLNFVKKSSAA NPA EAG PIVVHCSAgVGRTGTYI
Hum_LCA_D1 RQFQFMAwPDH G VPEYPTPILAFLRRVKAC NPL DAG PMVVHCSAgAGRTGCFI
Hum_PTP_mu_D1 RQFHFTGwPDH G VPYHATGLLGFVRQVKSK SPP SAG PLVVHCSAgAGRTGCFI
Hum_PTP_alpha_D1 TQFHFTSwPDF G VPFTPIGMLKFLKKVKAC NPQ YAG AIVVHCSAgVGRTGTFV
Hum_PTP_opsilon_D SQLHFTSwPDF G VPFTPIGMLKFLKKVKTL NPV HAG PIVVHCSAgVGRTGTFI
Mouso_CD45_D1 THIQFTSwPDH G VPEDPHLLLKLRRRVNAF SNF FSG PIVVHCSAgIGRTGTYI
Hum_SH.PTP2 WQYHFRTwPDH G VPSDPGGVLDFLEEVHHK QESIMDAG PVVVHCSAgIGRTGTFI
Hum_SH.PTP1 WHYQYLSwPDH G VPSEPGGVLSFLDQINQR QESLPHAG PIIVHCSAgIGRTGTII
Hum_PTP_bola RHFHYTVwPDH G VPETQSLIQFVRTVRDY INRSPGAG PTVVHCSAgVGRTGTFI
Dros_PTP10D RHFHFTTwPDF G VPNPPQTLVRFVRAFRDR ICA EQR PIVVHCSAgVGRSGTFI
Hum_SAP.1 RQFHYQAwPDH G VPSSPDTLLAFWRMLRQW LDQTMEGG PPIVHCSAgIGRTGCFI
Ral_PTP_STEP KHYWFTSwPDQ K TPDRAPPLLHLVREVEEAAQQEGPHCS PIIVHCSAgIGRTGCFI
Dros_PTP69A_D1 TQYHYLTwKDF M APEHPHGIIKFIRQINSVYSLQ RG PILVHCSAgVGRTGTLV
Hum_MEG2 THFQFLSwPDY G VPSSAASLIDFLRVVRNQQSLAVSNMGARSKGQCPEPPIVVHCSAgIGRTGTFC
Hum_PTP.PEST YQFHYVNwPDH D VPSSFDSILDMISLMRKYQEHE DV PICIHCSAgCGRTGAIC
Hum_PTPH1 THLQYVAwPDH G IPDDSSDFLEFVNYVRSLRVDSE PVLVHCSAgIGRTGVLV
Dici_PTP1 TQYQYEGwPDH N IPDHTQPFRQLLHSITNRQNQIIPSSD RNVPITVHCSAgVGRTGTFC
Fiss_yeast_pyp1 HHYQYPNwSDC N SPENVKSMVEFLKYVNNSHGSG NTIVHCSAgVGRTGTFI
Fiss_yeast_pyp2 HHFWVHTwFD K THPDIESITGLIRCIDKVPNDG PMFVHCSAgVGRTGTFI
Hum_PTP_xi_D2 TQYHYTQwPDM G VPEYSLPVLTFVRKAAYA KRH AVG PVVVHCSAgVGRTGTYI
Hum_LCA_D2 RQFQFTDwPEQ G VPKTGEGFIDFIGQVHKT KEQFGQDG PITVHCSAgVGRTGVFI
Hum_PTP_alpha_D2 RQFHFHGwPEV G IPSDGKGMISIIAAVQKQ QQQ SGNH PITVHCSAgAGRTGTFC
Hum_PTP_opsilon_D2 RQFHFHGwPEI G IPAEGKGMIDLIAAVQKQ QQQ TGNH PITVHCSAgAGRTGTFI
Hum_PTP_mu_D2 QQFQFLGwPMYRD TPVSKRSFLKLIRQVDKWQEEYNGGEG PTVVHCLNgGGRSGTFC
Mouse_CD45_D2 YQYQCTTwKGE E LPAEPKDLVSMIQDLKQKLPKASPEGMKYH KHASILVHCRDgSQQTGLFC
Dros_PTP69A_D2 TQFQYNGwPTVDGEVPEVCRGIIELVDQAYNHYKNNKNSGC RSPLTVHCSLgTDRSSIFV
Hum_PTP_zeia_D2 RHFQCPKwPN PDSPISKTFELISVIKEEAANR DG PMIVHDEHGGVTAGTFC
Hum_PTP_gamma_D2 RHFQCPKwPN PDAPISSTFELINVIKEEALTR DG PTIVHDEYGAVSAGMLC
Dros_PTP99A_D2 KMLHCPSwPEM SNPNSIYDFIVDVHERCNDY RNG PIVIVDRYGGAQACTFC
Yarsinia_PTP PVVHVGNwPDQTAVSSEVTKALASLVDQTAETKRNMYESKGSSAVADDSKLRPVIHCRAgVGRTAQLI
PTP1Bseq.no. 180 190 200 210 220
PTP1B66
β9 α3 β10
Figure 1D

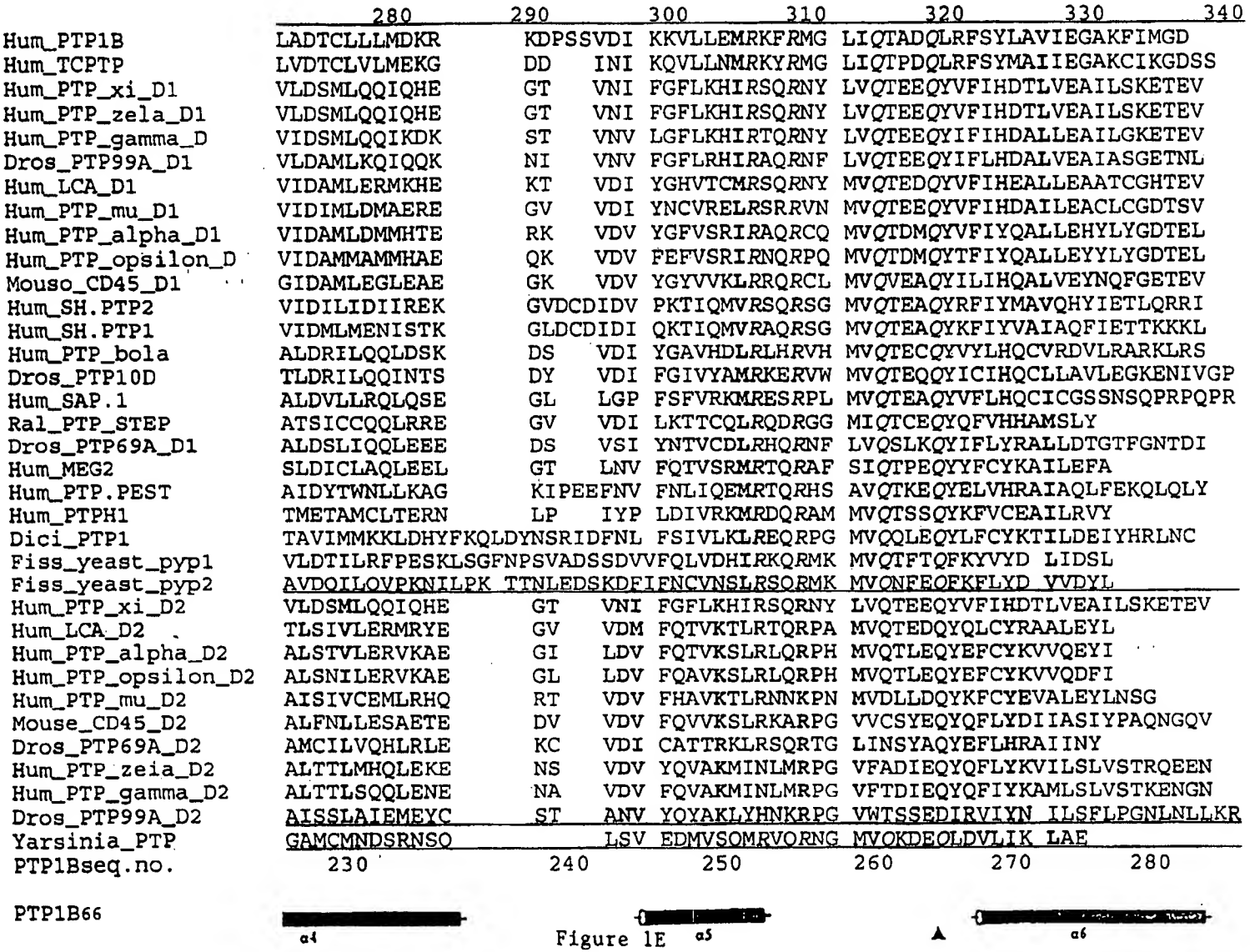
280 290 300 310 320 330 340
Hum_PTP1B LADTCLLLMDKR KDPSSVDI KKVLLEMRKFRMG LIQTADQLRFSYLAVIEGAKFIMGD
Hum_TCPTP LVDTCLVLMEKG DD INI KQVLLNMRKYRMG LIQTPDQLRFSYMAIIEGAKCIKGDSS
Hum_PTP_xi_D1 VLDSMLQQIQHE GT VNI FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_PTP_zela_D1 VLDSMLQQIQHE GT VNI FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_PTP_gamma_D VIDSMLQQIKDK ST VNV LGFLKHIRTQRNY LVQTEEQYIFIHDALLEAILGKETEV
Dros_PTP99A_D1 VLDAMLKQIQQK NI VNV FGFLRHIRAQRNF LVQTEEQYIFLHDALVEAIASGETNL
Hum_LCA_D1 VIDAMLERMKHE KT VDI YGHVTCMRSQRNY MVQTEDQYVFIHEALLEAATCGHTEV
Hum_PTP_mu_D1 VIDIMLDMAERE GV VDI YNCVRELRSRRVN MVQTEEQYVFIHDAILEACLCGDTSV
Hum_PTP_alpha_D1 VIDAMLDMMHTE RK VDV YGFVSRIRAQRCQ MVQTDMQYVFIYQALLEHYLYGDTEL
Hum_PTP_opsilon_D VIDAMMAMMHAE QK VDV FEFVSRIRNQRPQ MVQTDMQYTFIYQALLEYYLYGDTEL
Mouso_CD45_D1 GIDAMLEGLEAE GK VDV YGYVVKLRRQRCL MVQVEAQYILIHQALVEYNQFGETEV
Hum_SH.PTP2 VIDILIDIIREK GVDCDIDV PKTIQMVRSQRSG MVQTEAQYRFIYMAVQHYIETLQRRI
Hum_SH.PTP1 VIDMLMENISTK GLDCDIDI QKTIQMVRAQRSG MVQTEAQYKFIYVAIAQFIETTKKKL
Hum_PTP_bola ALDRILQQLDSK DS VDI YGAVHDLRLHRVH MVQTECQYVYLHQCVRDVLRARKLRS
Dros_PTP10D TLDRILQQINTS DY VDI FGIVYAMRKERVW MVQTEQQYICIHQCLLAVLEGKENIVGP
Hum_SAP.1 ALDVLLRQLQSE GL LGP FSFVRKMRESRPL MVQTEAQYVFLHQCICGSSNSQPRPQPR
Ral_PTP_STEP ATSICCQQLRRE GV VDI LKTTCQLRQDRGG MIQTCEQYQFVHHAMSLY
Dros_PTP69A_D1 ALDSLIQQLEEE DS VSI YNTVCDLRHQRNF LVQSLKQYIFLYRALLDTGTFGNTDI
Hum_MEG2 SLDICLAQLEEL GT LNV FQTVSRMRTQRAF SIQTPEQYYFCYKAILEFA
Hum_PTP.PEST AIDYTWNLLKAG KIPEEFNV FNLIQEMRTQRHS AVQTKEQYELVHRAIAQLFEKQLQLY
Hum_PTPH1 TMETAMCLTERN LP IYP LDIVRKMRDQRAM MVQTSSQYKFVCEAILRVY
Dici_PTP1 TAVIMMKKLDHYFKQLDYNSRIDFNL FSIVLKLREQRPG MVQQLEQYLFCYKTILDEIYHRLNC
Fiss_yeast_pyp1 VLDTILRFPESKLSGFNPSVADSSDVVFQLVDHIRKQRMK MVQTFTQFKYVYD LIDSL
Fiss_yeast_pyp2 AVDQILQVPKNILPK TTNLEDSKDFIFNCVNSLRSQRMK MVQNFEQFKFLYD VVDYL
Hum_PTP_xi_D2 VLDSMLQQIQHE GT VNI FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_LCA_D2 TLSIVLERMRYE GV VDM FQTVKTLRTQRPA MVQTEDQYQLCYRAALEYL
Hum_PTP_alpha_D2 ALSTVLERVKAE GI LDV FQTVKSLRLQRPH MVQTLEQYEFCYKVVQEYI
Hum_PTP_opsilon_D2 ALSNILERVKAE GL LDV FQAVKSLRLQRPH MVQTLEQYEFCYKVVQDFI
Hum_PTP_mu_D2 AISIVCEMLRHQ RT VDV FHAVKTLRNNKPN MVDLLDQYKFCYEVALEYLNSG
Mouse_CD45_D2 ALFNLLESAETE DV VDV FQVVKSLRKARPG VVCSYEQYQFLYDIIASIYPAQNGQV
Dros_PTP69A_D2 AMCILVQHLRLE KC VDI CATTRKLRSQRTG LINSYAQYEFLHRAIINY
Hum_PTP_zeia_D2 ALTTLMHQLEKE NS VDV YQVAKMINLMRPG VFADIEQYQFLYKVILSLVSTRQEEN
Hum_PTP_gamma_D2 ALTTLSQQLENE NA VDV FQVAKMINLMRPG VFTDIEQYQFIYKAMLSLVSTKENGN
Dros_PTP99A_D2 AISSLAIEMEYC ST ANV YQYAKLYHNKRPG VWTSSEDIRVIYN ILSFLPGNLNLLKR
Yarsinia_PTP GAMCMNDSRNSQ LSV EDMVSQMRVQRNG MVQKDEQLDVLIK LAE
PTP1Bseq.no. 230 240 250 260 270 280
PTP1B66
α1
α5
α6

Figure 1E

Vmax and Km of 37kDa-PTP1B Mutants Toward RCML

Enzyme	Vmax (nmol/min/mg)	Km (nM)	Kcat (min^{-1})
wild type	60200	102	2244
Tyr 46 → S	4120	1700	154
→ L	4160	1700	155
Glu 115 → A	5700	45	212
→ D	5900	20	220
Lys 116 → A	68600	150	2557
Lys 120 → A	19000	80	708
Asp 181 → A	0.61	≤126	0.023
→ E	97	10	3.6
His 214 → A	700	20	26
Cys 215 → S	0.026		0.00097
Arg 221 → K	11	80	0.41
→ M	3.3	1060	0.12
Gln 262 → A	720	9	27

Figure 2